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13. ABSTRACT *(Maximum 200)*

Retrotransposable elements comprise as much as 10-15% of mammalian genomes. Several of these elements possess the ability to relocate within the host's genome and thereby constitute a mechanism for genetic mutation leading to disease. Mouse mammary carcinomas of various etiologies show upregulation of two classes of retrotransposable elements, intracisternal a particles (IAPs) and murine viral leukemia elements (MuLVs). Expression of MuLVs has lead to the appearance of novel restriction fragments associated with the ecotropic MuLV in D2 HANs and several D2 tumors from BALB/c mice. Cloning flanking sequences to several integration sites and genetic mapping of a subset has suggested integration events have occurred in the D2 HANs and tumors. Characterization of one integration site, EN31, on chromosome 6 in the mouse, has identified a 140 kDa G-protein activating protein (GAP) for the small G-protein Rho. The RhoGAP was overexpressed in D2 HANs and tumors, D1 non-transformed and transformed cell lines, and fC3H mammary carcinomas. Expression of p140mRhoGAP in DMBA-induced primary mouse mammary carcinomas was reduced suggesting a role in *ras* dependent tumorigenesis. p140mRhoGAP was expressed most abundantly in the brain and in the developing nervous system. The p140mRhoGAP was localized to the cytoplasm adjacent to the plasma membrane and was inhibitory to actin stress fiber formation.

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PI - Signature Date

Table of Contents

Front Cover	1.
Standard Form (SF) 298	2.
Foreword	3.
Table of Contents	4.
Introduction	5.
Training/Research Results	5.
IAPs v. MuLVs	8.
References	9.
Appendix I (Appendix to the Summary)	10.
Appendix II (Research Data Figures)	11.

Introduction

Retrotransposons reside in the genomes of such diverse organisms as yeast (Ty elements), Drosophilia (penelope elements), mice (intracisternal A particles [IAP], murine viral leukemia related elements[MuLVs]) and humans (LINEs, SINEs, and human endogenous retroviral like elements[HERVs]). It has been postulated that endogenous retroviral like elements and retroposons constitute as much as 15% of the human genome (Kazazian, 1999). A very small percentage of these elements maintain their capacity for reverse transcription from the endogenous provirus and reinsertion into the host's genome, possibly resulting in altered gene expression. Indeed, several such mutations have been described for both mice and humans, for example an IAP insertion into the agouti locus of the mouse and a LINE-1 insertion into the *myc* gene in a human breast carcinoma (Michaud et al. 1994; Morse et al. 1988). In order to identify genes associated with conversion of normal mammary epithelium into mammary carcinoma, we attempted to isolate genes which had been dysregulated via a retrotransposition event. Integration of retrotransposons may alter gene expression and function in one of several ways; a) enhancer mutations whereby the integration event regulates gene transcription from some distance, b) integration promotes transcription directly from the powerful promoter contained with the retrotransposon's LTRs, c) incorporation of an LTR into the primary transcript which donates poly adenylation signals resulting in premature truncation of the gene, and d) incorporation of an LTR into a transcript thereby introducing extragenic sequences which would then alter the downstream reading frame. Most genes altered by retroviral insertion were identified more or less accidentally by isolating the mutant or altered gene and discovering a retrotransposon was responsible for misregulation. Previously, our group reported overexpression of IAPs in primary mouse mammary carcinomas compared to all stages of mammary gland development (Asch, 1993). We, therefore, adopted the screening protocol of Mager and colleagues (1992) which utilized probes for the U3 and U5 regions of the LTRs to isolate retrotransposon (IAP)/cellular gene chimeric molecules directly.

Results Summary

The original proposal promised the isolation of chimeric molecules between intracisternal a particles (IAPs) and cellular transcripts involved in the progression of mammary carcinomas. A cDNA library was constructed and differentially screened with probes for the U3 and U5 regions of the LTRs and the *env* gene of IAP. Clones positive for U3 and/or U5 hybridization but negative for the env gene were characterized by sequence analysis. Several clones contained normal cellular sequences chimeric with the U3 IAP LTR, however two poly A signals (AAUAAA) and poly A tails were present within the clones. One of these, an ~2.2kb clone, hybridized to two transcripts on Northern analysis, a 6.6 kb transcript observed in all tissues examined and a 1.4 kb transcript detected in DMBA-induced mouse mammary carcinomas. Sequence analysis revealed homology with IAP through part of the *env* gene, splicing factor U1 specific protein C, a murine B1 repeat element and regions with no significant homology with any genes within the data base. First attempts to isolate the full length tumor associated transcript isolated only U1 specific protein C and several unrelated cDNA transcripts. Subsequent screening normal mouse brain, kidney and liver cDNA libraries isolate several clones which hybridized to the correct transcripts on Northern blots however each clone identified had only the murine B1 element in common. No other sequence similarity was present. A 1.8kb clone was isolated from the mouse kidney cDNA library using probes derived from the unique sequence of the original isolate. This clone was used as the basis for 5' RACE experiments to acquire the full length transcript from a Marathon RACE kit produced from mouse kidney. Three extensions of the gene were isolated, all overlapped in sequence by approximately 150bp, but only two continued to hybridize to the original two transcripts upon Northern analysis. The 5' most extension hybridized to transcripts of approximately 1.8 and 2.0 kb. Genetic mapping experiments using the Jackson labs

([C57Bl/6 X Spretus] X Spretus) backcross had localized the original clone to mouse chromosome 17 while the kidney clone was localized to mouse chromosome 7. Southern analysis using either the original or the kidney clone failed detect mutations between normal mammary gland and tumors utilizing several different restriction enzymes. This, in addition to the data above (specifically the several clones from various cDNA libraries which hybridized to the same transcripts on Northern blots which had only a murine B1 element in common) led us to conclude the hybridization detected was most likely due to the presence of the murine B1 element. Therefore it was the decision of my academic advisory committee to drop the project as it would be insufficient to constitute a viable thesis.

Previously, our lab discovered up regulation of the ecotropic MuLV in preneoplastic tissues (hyperplastic alveolar nodules, HANs) and mammary tumors induced by various etiologies (Asch et al., 1993). In addition, several distinct restriction fragments had been associated with the ecotropic MuLV in these same HANs and tumors by Southern analysis (Natoli et al., 1996; M. R. Crowley, this report). Inverse PCR was used to isolate the flanking sequences adjacent to the ecotropic MuLV integrations (Slon et al, in prep). Sequence comparisons between the isolated flanking sequences and the endogenous ecotropic MuLV locus (*Cv-1* on mouse chromosome 5, Kozak and Rowe. 1979) suggested new positions within the genome. Two clones contained the characteristic 4 bp duplication of DNA directly adjacent to the provirus which suggests proviral integration as opposed to other genetic events (Slon et al., in prep). Furthermore, several of the integration events were localized within the mouse genome by genetic backcross analysis using the flanking sequences as markers. Shown in figure 1 are the genetic localizations of 4 of the 8 clones isolated, EN21, EN31 (p140mRhoGAP), D21b and D25a. Integration sites EN21 and D25a occurred on mouse chromosome 13 at the distal and proximal ends respectively (Fig. 1). The D21b integrant was localized to mouse chromosome 2 near the mouse prion protein gene (Fig 1.). Integration of the ecotropic MuLV, EN31, was in the distal end of mouse chromosome 6 (Fig. 1). Sequences directly adjacent to the proviruses for EN21 and EN31 were used to screen a mouse BAC library. Several BAC clones were isolated representing both loci. Shotgun cloning and random sequencing of clones revealed sequences derived from the EN31 BACs which were homologous to a human transcript, KIAA0411 (data not shown). Using the extreme 3' end of the human gene 2 mouse ESTs were identified by homology searches. PCR analysis of the BAC clones with primers directed against the exon identified in the original search and primers specific to the mouse ESTs demonstrated the exon and the extreme 3' end of the clone were contained on the same BAC (data not shown). These data suggested that the coding sequences for the mouse homologue to human KIAA0411 were included on the BACs. Reverse-transcription PCR and 5' RACE techniques generated the full length mouse cDNA (Fig. 2).

The murine Kiaa0411 gene is approximately 85% homologous to the human KIAA0411 transcript found in the database. Sequence analysis of the murine gene reveals an 8.4 knt transcript with an approximate 460bp 5' UTR, a 2.9 knt ORF and an approximately 5 kb 3' UTR. The coding sequence is highly homologous to the human gene at both the nucleotide (~90%) and amino acid (92% identity) levels. Interestingly, the 3' untranslated regions of the human and mouse transcripts retain sequence homology (~85%) (data not shown). The 2.9 knt open reading frame encodes a predicted protein of 963 amino acids containing regions homologous to Rho activating protein (RhoGAP) and src homology-3 (SH3) domains (Figs. 2 and 3 B and C). In addition, the amino terminus contains limited homology (46% identity) to another RhoGAP, RGC1, expressed primarily in human hematopoietic tissues (Tribioli et al, 1996) (Fig. 3A). SDS-PAGE analysis of a His6 tagged protein expressed in bacteria resulted in a peptide of approximately 140 KDa (Fig 4). We now propose the name p140mRhoGAP for the murine homologue to hKIAA0411.

Since p140mRhoGAP was isolated as a result of an ecotropic MuLV integration we assayed for expression in mouse mammary hyperplasias and carcinomas. Southern analysis confirmed the ecotropic MuLV integration into the p140mRhoGAP locus in several D2 HANs and D2 tumors (Fig 5). The ecotropic MuLV integration occurred within

an intron (data not shown) and therefore could either promote transcription from the LTR or act as an enhancer on the endogenous promoter. Northern analysis of total RNA on normal mammary glands from a virgin mouse, D2 HANs and D2 tumors with a probe from the 3' end of p140mRhoGAP detected only the 8.4 knt transcript (Fig.6). The transcript was overexpressed from 2.3 to 7.5 fold when compared to normal virgin mammary glands (Fig. 6). These data suggest the ecotropic MuLV integration was acting as an enhancer. To confirm the enhancer effect BALB/c D1 cell lines were screened for the presence of an ecotropic MuLV integrant at the p140mRhoGAP locus. The D1 mammary cell lines, CL-S1, -SA and +SA were derived from a HAN isolated from a BALB/c mouse. The cells are in varying stages of transformation (CL-S1 cell are nontransformed, -SA cells are transformed but do not form colonies in a soft agar assay, +SA cells are transformed and form colonies in soft agar). Southern analysis on -SA and +SA cells did not detect any new restriction fragments associated with the ecotropic MuLV (Figs. 7, CL-S1 were not assayed at the time of writing as we lost the cell line due to bacterial contamination). p140mRhoGAP was, however, overexpressed in all three cell types compared to the normal virgin mammary gland (Fig. 8). Indeed, expression in the nontransformed cell line, CL-S1, was actually higher than in the two transformed cell lines (Fig. 8). Additionally, several other mouse mammary tumors were screened for upregulation of p140mRhoGAP. Mammary carcinomas from BALB/c-fC3H mice (MMTV-induction) showed upregulation of p140mRhoGAP (Fig. 9). In contrast, mammary tumors from BALB/c mice treated with DMBA (chemical carcinogen) did not overexpress p140mRhoGAP (Fig. 9). These results, in addition to those above suggest that upregulation of p140mRhoGAP occurs in hormonally and virally induced mouse mammary carcinomas independent of ecotropic MuLV status at the locus and that chemical carcinogenesis failed to increase expression of the gene.

To determine the normal distribution of p140mRhoGAP a multiple tissue Northern was screened. Shown in figure 10 A, p140mRhoGAP expression was highest in the adult brain with detectable levels in the virgin mammary gland. Upon longer exposure of the autoradiogram p140mRhoGAP expression was detected in most other tissues at very low levels, the exception was white adipose tissue where expression was not detected (expression was also not observed using RT-PCR, data not shown). Dissection of the brain into three regions, cerebral cortex, mid brain and cerebellum showed p140mRhoGAP expression in all regions, however expression in the cerebellum was higher compared to the other two (Fig. 10 C). The tissue distribution of p140mRhoGAP would suggest a prominent role for the protein in development of the nervous system. Expression of the gene was detected in whole embryos at the earliest time point examined, E11.5 (Fig. 11). Separating the developing head from the body resulted in very high levels of expression in the head at E13.5, peaking around E15.5 and decreasing slightly through birth (Fig. 11). Expression levels in the bodies of the same embryos showed low levels of expression at E13.5 with expression steadily decreasing through E17.5, where expression is almost undetectable (Fig. 11). In the mammary gland expression was approximately the same in glands from virgin and pregnant animals with decreased expression in lactation and involution (Fig. 9). The decrease in expression of p140mRhoGAP during lactation may be due to increases in milk gene synthesis (i.e. WAP and casein) as many genes appear downregulated during lactation. p140mRhoGAP was detected in both the epithelial and stromal compartments of the virgin mammary gland by RT-PCR analysis (data not shown).

The ras superfamily of proteins, including ras, rho, arf, ran, rab and sar, become activated through their association with GTP and are involved in such diverse functions as cellular growth (ras), cytoskeletal reorganization (rho, rac and cdc42hs) and vesicular trafficking (ran) (Zohn et al., 1998). Ras family members are membrane bound through farnesylation and palmitoylation at the carboxy terminus and membrane association is required for activity (Magee and Marshall, 1999). Ras members have very low intrinsic GTPase activity and the transition from the active form, Ras-GTP, to the inactive form, Ras-GDP, is catalyzed by activating proteins, RasGAPs. It has been proposed that GAPs reside in the cytoplasm and are translocated to the membrane upon activation (Lamarche

and Hall, 1994). To assess intracellular localization of p140mRhoGAP a RhoGAP/green fluorescent protein fusion was constructed and transiently transfected into COS-7 cells. Visualization of the GFP fluorescence demonstrated both cytoplasmic and membrane localization for p140mRhoGAP (Fig. 12). The small G-protein Rho is responsible for the actin stress fiber formation in response to various stimuli (Ridley, 1996). Stable introduction of p140mRhoGAP into COS-7 cells followed by stimulation of actin stress fiber formation resulted in a lack of stress fibers in cells expressing p140mRhoGAP compared to vector only controls (Fig. 13). These data suggest that p140mRhoGAP is acting as a G-protein activating protein and one effector is the small GTP-binding protein Rho.

IAPs v. MuLVs

The original statement of work had outlined the cloning and characterization of transcripts which were altered by intracisternal a particle retrotransposition associated with mammary carcinomas. Briefly, I had proposed to clone RNA transcripts which were chimeric with IAP sequences, determine the nature of the mutation (see Introduction) and isolate the chimera's normal counterpart. In addition, expression of the gene transcript at the RNA level was to be conducted on several normal tissues, preneoplasias and tumors derived through various means (chemical, viral and hormonal). Following the assessment of the gene's expression profile, sequence and functional analysis was proposed to identify potential mechanisms of action for both the normal and mutated gene. Analysis of the integration site within the genomic DNA would have provided evidence for IAP's role in tumor progression in regards to the isolated mutation. Transformation of benign mammary epithelial cells to a more malignant phenotype through anchorage independent growth and/or tumor formation in immunocompromised mice were to be done with the mutant gene. Ultimately these results would address the carcinogenic potential of the chimera.

In the present report I have summarized the cloning and characterization of a novel murine RhoGAP isolated due to the integration of an ecotropic MuLV from BALB/c D2 mammary carcinomas. Many aspects of the work described above for MuLV follow the statement of work as outlined. Indeed, I have cloned and characterized a gene into which a retrotransposon had integrated (MuLV integration at the p140mRhoGAP locus on mouse chromosome 6). The normal genomic locus was cloned and analysis was conducted to determine the nature of the retrotransposition event (the MuLV integrated into an intron and was possibly acting as an enhancer to upregulate gene transcription). The full length gene was cloned and sequence analysis suggested a potential role in cellular physiology (GTPase-activating protein for the small G-protein binding protein Rho). A mutant or chimeric transcript was not isolated as the integration did not promote or truncate the normal transcript. However, overexpression was observed in the tumors bearing the integrated MuLV. Therefore, the mutation that was caused by the retrotransposition event appeared to be an enhancer mutation affecting the normal regulation of a cellular gene. Unfortunately, the extent of MuLV's role in upregulation of p140mRhoGAP is questionable as other tumors and hyperplasias which also overexpressed the gene did not contain an ecotropic MuLV at the same locus. Indeed, the +SA and -SA cell lines which have upregulation of p140mRhoGAP, do not harbour any novel ecotropic MuLV integrations. The normal tissue distribution of the RhoGAP was identified along with an abbreviated developmental profile (also proposed in the original application). The normal function of the isolated gene was investigated circumstantially by intracellular localization and actin reorganization assays. Finally, at the time of writing, benign human mammary epithelial cell lines (184A1N4 and MCF10A cells do not express p140mRhoGAP, data not shown) had been stably transfected with the p140mRhoGAP and were in the selection phase following which cellular transformation assays would be performed. In conclusion, I feel the goals of the project have been met with ecotropic MuLV as the mutagen and not IAP.

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Appendix I

Key Research Accomplishments

- Identification and chromosomal localizations of new ecotropic murine viral leukemia elements associated with mouse mammary carcinomas.
- Genomic cloning of two MuLV integration sites.
- Identification and isolation of a gene associated with an ecotropic MuLV integration.
- Cloning and sequence analysis of a novel mouse RhoGAP, p140mRhoGAP.
- Determination of p140mRhoGAP's expression profile in mouse mammary carcinomas with and without an ecotropic MuLV insertion.
- Determination of p140mRhoGAP's normal tissue distribution.
- Determination of the developmental expression profile of p140mRhoGAP.
- Analysis of intracellular localization of p140mRhoGAP by GFP fusion.
- Assessment of p140mRhoGAP's role in cytoskeletal reorganization and possible tumor suppressor function.

Reportable Outcomes

- Abstract presented at the Gordon Research Conference on mammary gland biology, 1999.
- Abstract submitted to the 13th International Mouse Genome Conference, 1999.
- The degree of Doctor of Philosophy in Molecular and Cellular Biology granted by the State University of New York at Buffalo, Roswell Park Graduate Division, expected fall of 1999.
- Post doctoral interviews at Vanderbilt University, Nashville TN; University of Minnesota Cancer Center, Minneapolis, MN; University of Massachusetts at Amherst, Amherst, MA; The University of Cincinnati, Cincinnati, OH; The National Cancer Institute, Bethesda, MD.

Appendix II

Figure 1.

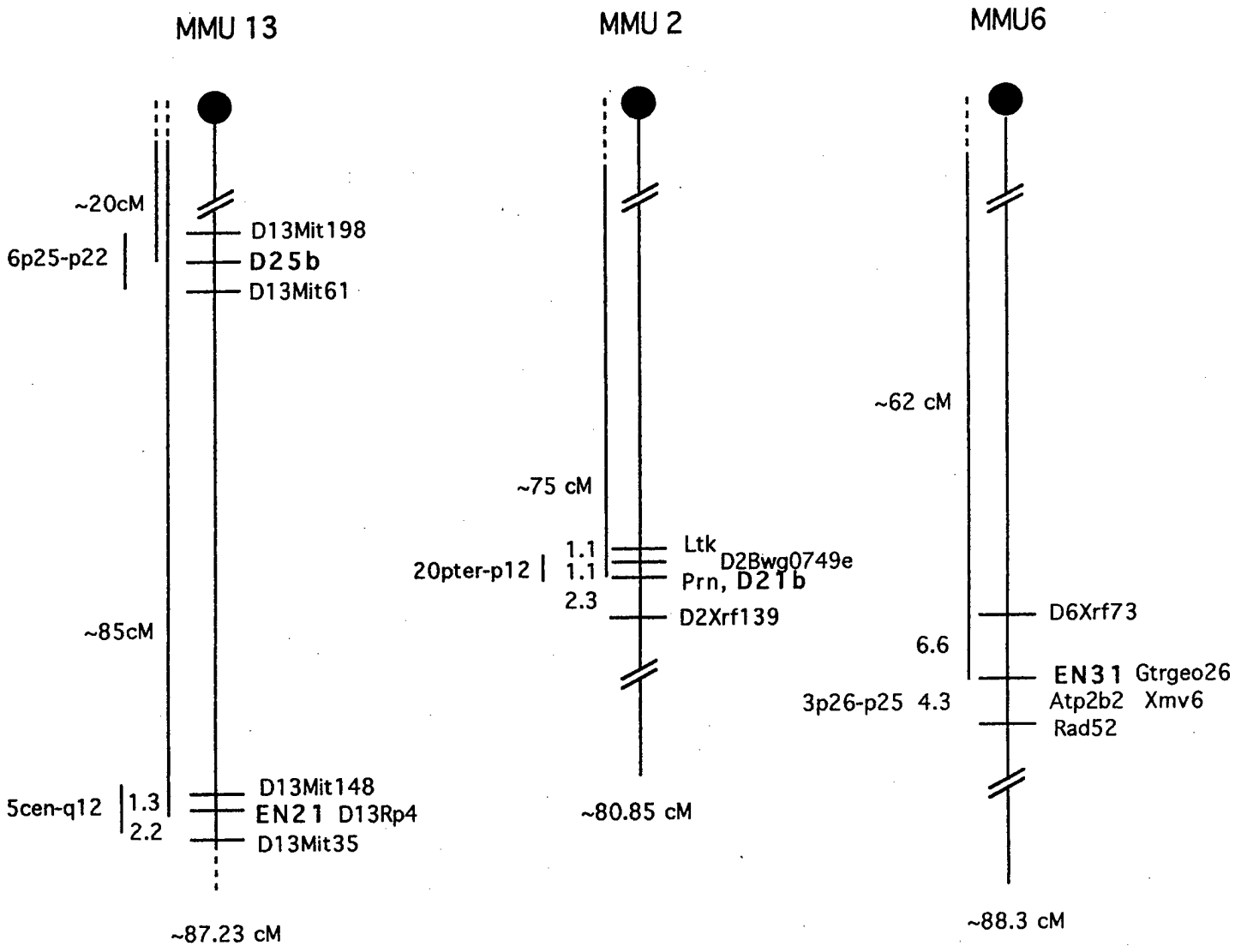


Figure 1. Chromosomal localizations of the ecotropic MuLV integration sites. The (C57Bl/6 X Spretus) X Spretus backcross pannel from the Jackson Labs was screened by PCR for EN21, D21b and D25b or by hybridization using a probe adajacent to the EN31 integration site. The data were compiled and analyzed using MapManager. Integration sites for EN21 and D25b were on distal and proximal ends of chromosome 13, respectively. The D21b integration site localized to chromosome 2 and EN31 (p140mRhoGAP) mapped to chromosome 6. The ecotropic MuLV integration sites are shown in bold. Map locations and distances are from the Jackson Labs composite maps for the various chromosomes. Recombination frequencies are given in cMs and the homologous region in the human genome are shown.

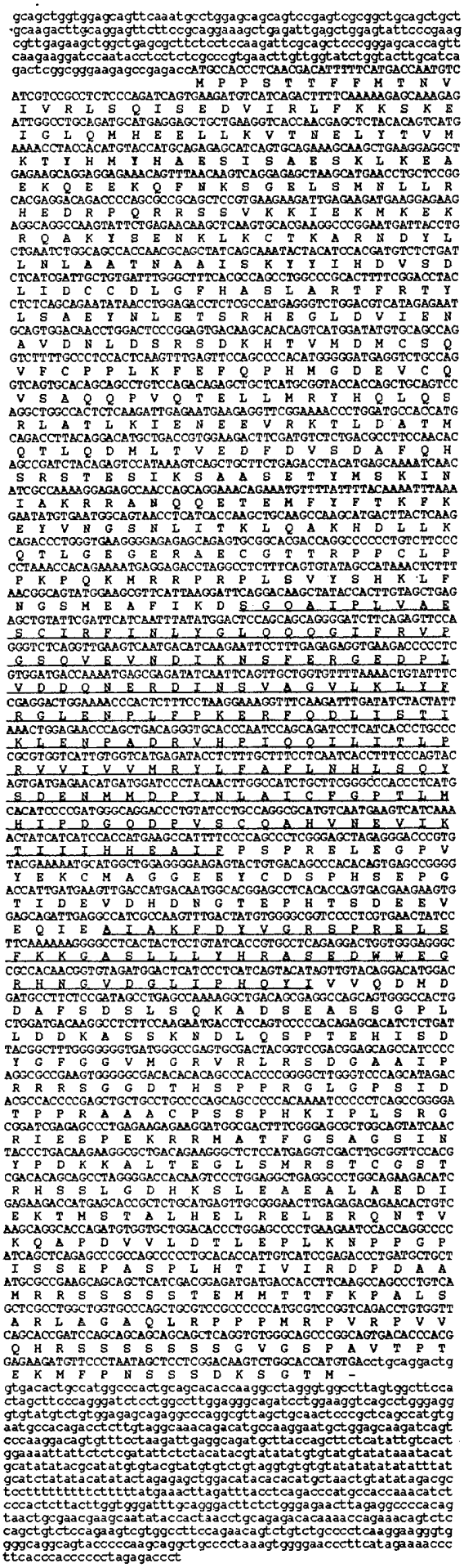
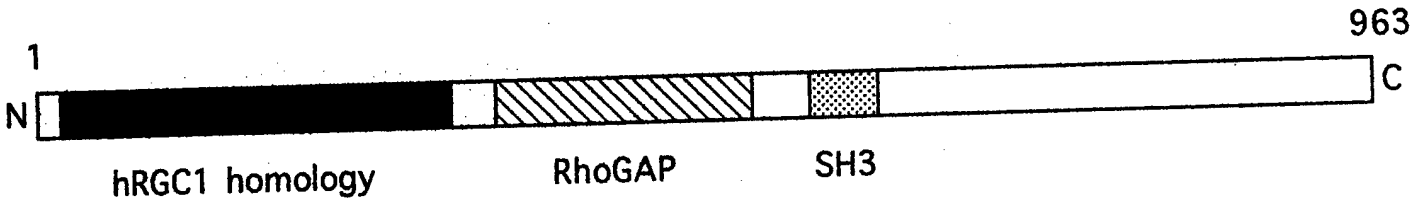


Figure 2.

Figure 2. Sequence analysis and primary amino acid translation for the cDNA encoding the mouse homologue of KIAA0411. For brevity only a portion of the 8.2 kb mouse cDNA sequence is shown. The sequence contains a 2.9 kb open reading frame which encodes for a 963 amino acid protein. The protein has a predicted molecular mass of 108Kda and a pI=5.99. The amino acid translation is in the single amino acid code. The RhoGAP and SH3 domains are underlined (see also Fig. 3). The 5' and 3' untranslated regions are in lower case while the ORF is in uppercase.

Figure 3.

A.



B.

```
mKiaa   385 SVYSHKLFNGSMEAFIKDSGQAIPLVAESCIRFINLYGLQQQGIFRVPGSQVEVNDIKNS 444
            S Y+ +LF G ME FI+ SGQ +PLV ESCIRFINL GLQ +GIFRV G+Q+ V++I+++
hRGC1   498 SQYNQRLFGGDMEKFIQSSGQPVPLVVESCIRFINLNGLQHEGIFRVSGAQLRVSEIRDA 557

mKiaa   445 FERGEDPLVDDQNERDINSVAGVLKLYFRGLENPLFPKERFQDLISTIKLENPADRVHPI 504
            FERGEDPLV+     D++SVAGVLKLYFR LE PLFP + F +L+++ +LE+ A+RV  +
hRGC1   558 FERGEDPLVEGCTAHDLDSVAGVLKLYFRSLEPPLFPPDLFGELLASSELEDTAERVEHV 617

mKiaa   505 QQILITLPRVVIVVMRYLFAFLNHLSQYSDENMMDPYNLAICFGPTLMHIPDGQDPVSCQ 564
             ++L  LP  V+VV+RYLF FLNHL+QYSDENMMDPYNLA+CFGPTL+ +P GQDPV+ Q
hRGC1   618 SRLLWRLPAPVLVVLRYLFTFLNHLAQYSDENMMDPYNLAVCFGPTLLPVPAGQDPVALQ 677

mKiaa   565 AHVNEVIKTIIIHHEAIF 582
              VN++++T+I+  + +F
hRGC1   678 GRVNQLVQTLIVQPDRVF 695
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C.

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mKiaa   637 AIAKFDYVGRSPRELSFKKGASLLLYHRASEDWWEGRHNGVDGLIPHQYI 686
            A+A F Y GR+ +ELSF++G  L L+ RAS DWW G HNG+ GLIPH+YI
hRGC1   751 AVACFAYTGRTAQELSFRRGDVLRLHERASSDWWRGEHNGMRGLIPHKYI 800
```

Figure 3. Cartoon and amino acid sequence homology of p140mRhoGAP. A). Schematic representation of the amino acid sequence of p140RhoGAP. The RGC1 homology domain is the solid box with the RhoGAP domain depicted by the stripped region and the SH3 homology domain denoted by the stippled box. B). Amino acid homology of the putative RhoGAP domain of p140mRhoGAP compared to the RhoGAP domain of the human RGC1. C). Amino acid similarities between the SH3 domain of p140mRhoGAP and the SH3 domain of the human RGC1 protein. The single amino acid code is used and pluses represent conserative changes between the two proteins.

Figure 4.

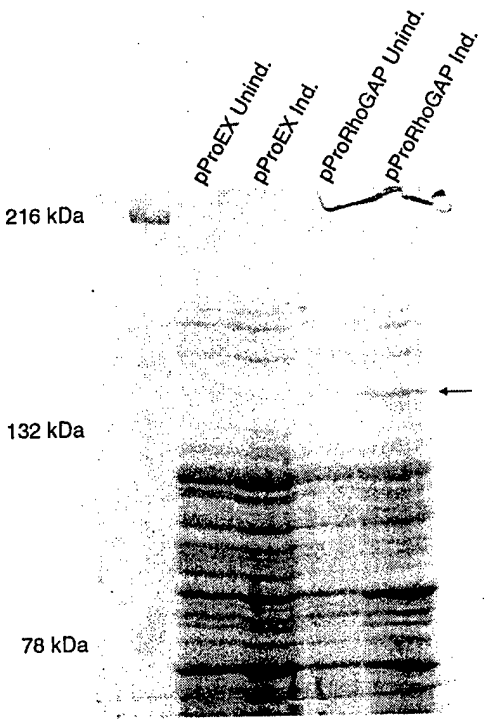

Figure 4. SDS-Polyacrylamide Gel Electrophoresis analysis of p140mRhoGAP. The coding region of p140mRhoGAP was placed downstream of an IPTG inducible promoter and bacteria were transformed with either the empty vector (pProEX) or p140mRhoGAP (pProRhoGAP). The cells were stimulated (pProEX Ind and pProRhoGAP Ind) with 0.6mM IPTG for 1.5 hours and harvested. Protein extracts from uninduced cells (pProEX Unind. and pProRhoGAP Unind.) and induced were seperated on a 6% SDS-PAGE gel and visualized with coomasie brilliant blue. The p140mRhoGAP is denoted wtih the arrow.

Figure 5.

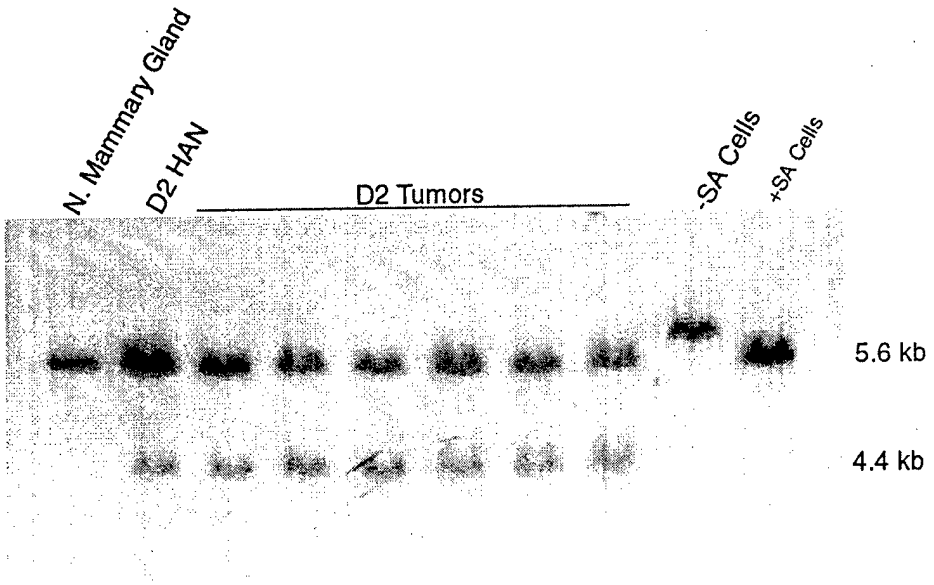

Figure 5. Southern analysis of the ecotropic MuLV integration event into the p140mRhoGAP locus. Genomic DNA was isolated from a mammary gland from a virgin mouse, D2 hyperplastic alveolar nodule (HAN), several D2 tumors, -SA cells and +SA cells, digested with *PvuII* and electrophoresed on a 0.8% agarose gel. The DNA was transfered to nylon membrane and hybridized with a 325 bp probe corresponding to the genomic region directly adjacent to the integration. The probe detects a 5.6 kb fragment from normal genomic DNA while the MuLV integration introduces a *PvuII* site and creates a 4.4 kb restriction fragment. The MuLV integration event has occurred in the D2 HAN as well as the D2 tumors. Disruption of the locus is not observed in the -SA or +SA cells (see text for details on these cell lines).

Figure 6.

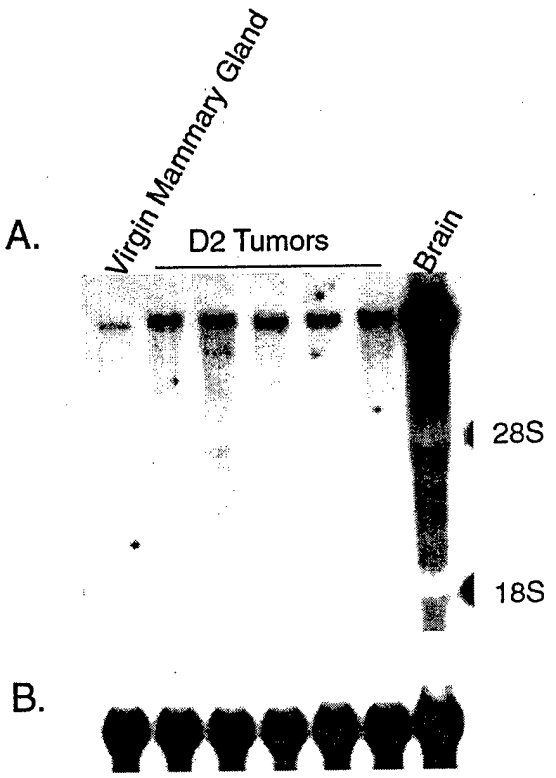

Figure 6. Northern analysis of D2 tumors with p140mRhoGAP. A). Total RNA was isolated from a mammary gland from a virgin mouse, several D2 tumors and whole mouse brain by TRI and electrophoresed through a 1.2%, 2.2M formaldehyde agarose gel. The RNA was transfered to nylon membrane and probed with an PCR generated fragment directed against the extreme 3' end of p140mRhoGAP. The probe detects an ~8.4 knt transcript in all tissues. The 28S and 18S ribosomal bands are indicated. B). Ribosomal DNA as a loading control.

Figure 7.

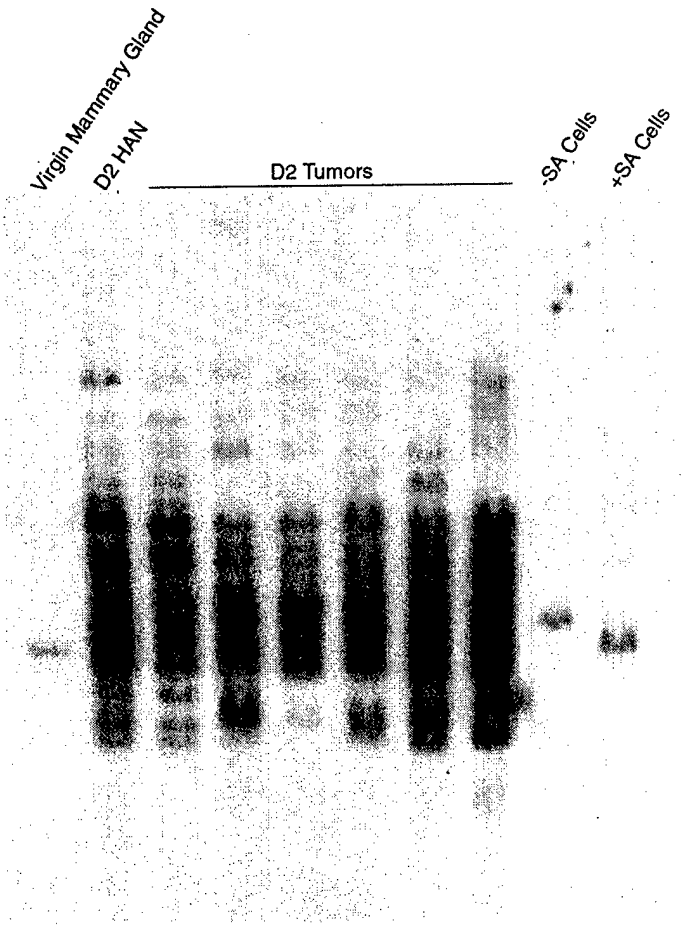

Figure 7. Southern analysis of MuLV integration sites in hyperplastic and neoplastic cells and tissues. The Southern blot described in Fig. 5 was stripped and reprobed with a 400 bp *Sma I* probe specific for the ecotropic MuLV. The *Sma I* probe detects a 4.2 kb *Pvu II* fragment which corresponds to the endogenous locus on chromosome 5. Note the plethora of novel restriction fragments associated with the ecotropic MuLV in the D2 HAN and tumors and the absence of new restriction fragments in the -SA and +SA cells.

Figure 8.

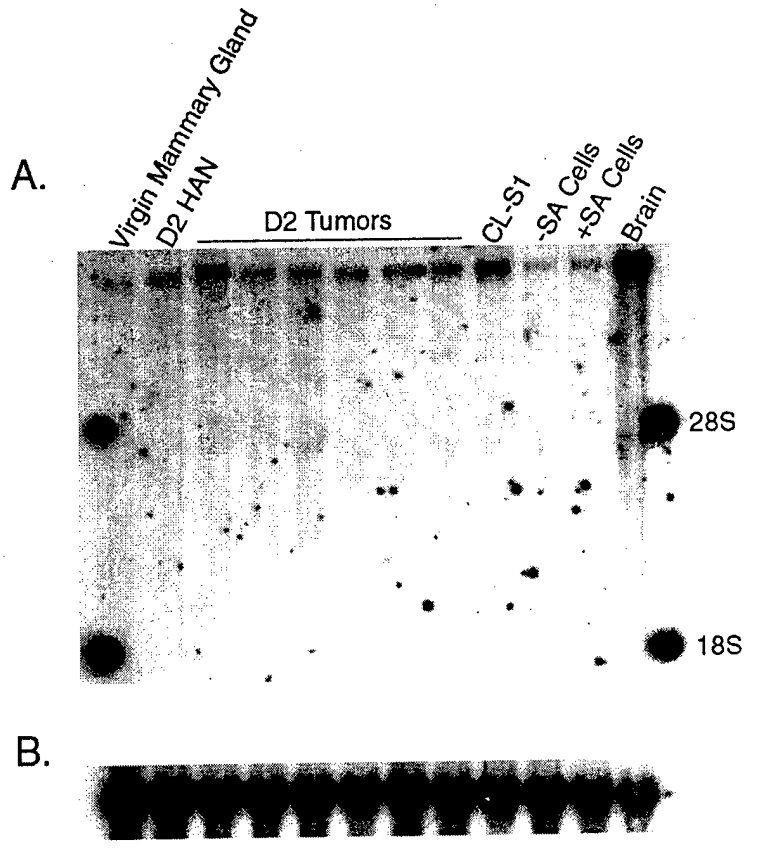

Figure 8. Northern analysis of D1 and D2 hyperplasias and neoplasias. A). Total RNA from normal mammary gland, D2 HAN, D2 tumors and CL-S1, -SA and +SA cells and normal mouse brain were isolated and processed as described in the legend to figure 6. For details on CL-S1, -SA and +SA cells see the main text. The 28S and 18S ribosomal bands are indicated. B). Ribosomal DNA probe as a loading control.

Figure 9.

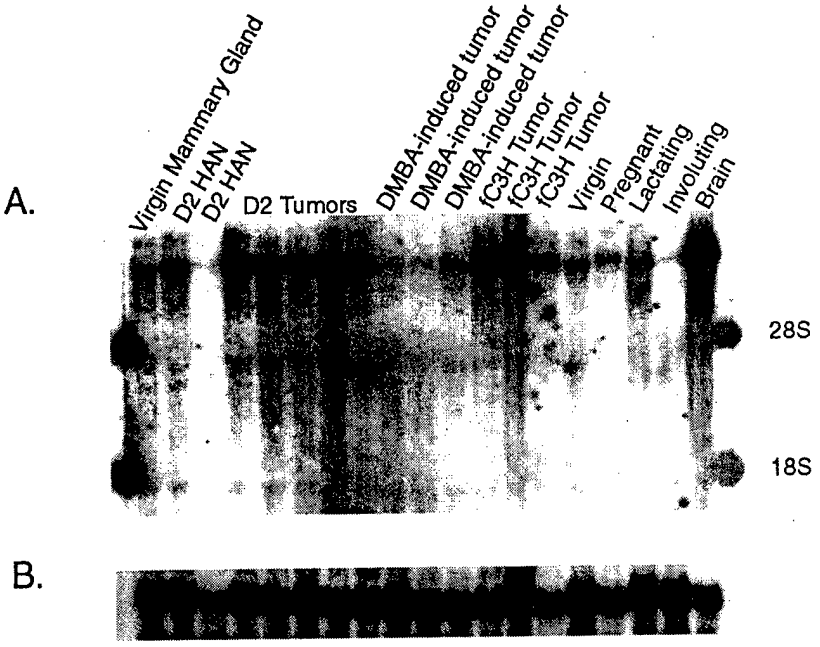

Figure 9. Northern analysis of mouse mammary tumors from various etiologies and normal mammary glands in different stages of development. Total RNA isolated from preneoplastic and tumors induced with hormonal (D2 HANs and tumors), chemical (DMBA-induced) or viral (fC3H) treatments were processed as described in the legend to figure 6. The D2 HAN sample in lane 3 was partially degraded (compare p140mRhoGAP signal to the rDNA signal in B). Note the reduction in message in the DMBA-induced tumors compared to the hormonally or virally induced tumors. The DMBA-induced tumors all contain an activating c-Ha-ras mutation at codon 61 (M. R. Crowley, unpublished results). The p140RhoGAP message is expressed at similar levels in mammary glands from virgin and pregnant animals but is reduced during involution. Decreases in expression levels in the lactating mammary gland may be due to milk gene expression causing a dilution effect. B.) Ribosomal DNA probe as a loading control.

Figure 10.

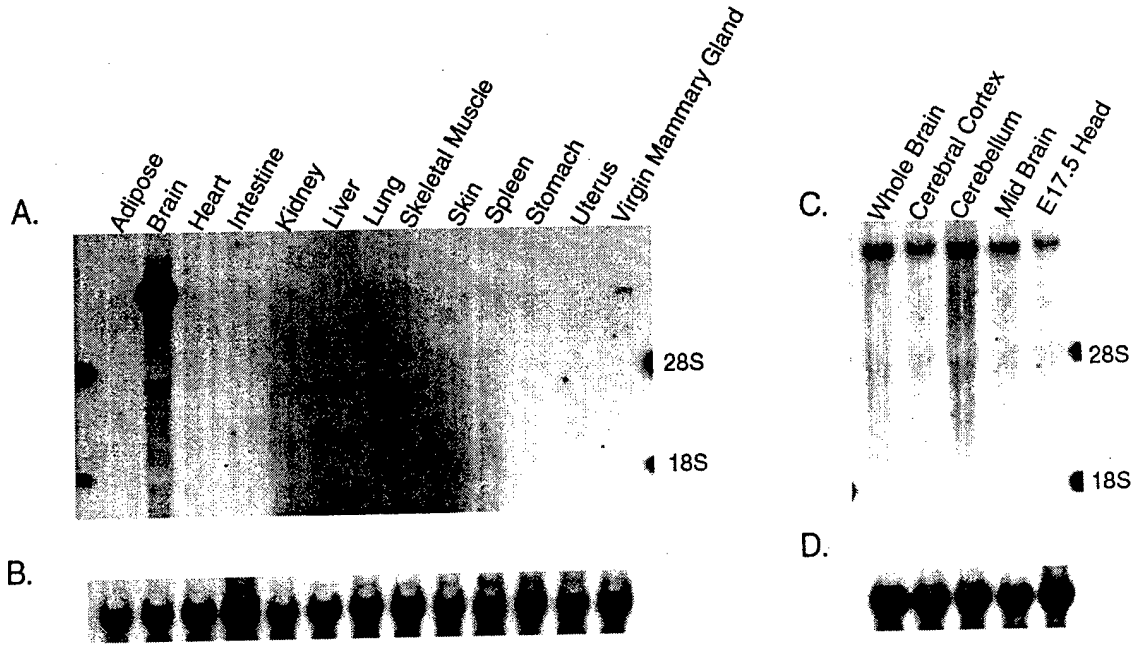

Figure 10. Northern analysis of mouse tissues and brain regions for the expression of p140mRhoGAP. A). Several adult tissues from a BALB/c mouse were removed and total RNA isolated. Expression of p140mRhoGAP is highest in the adult brain with detectable levels in heart, intestine, kidney, liver, lung, skeletal muscle, skin, spleen, stomach, uterus and virgin mammary glands. Expression was not detected in adipose tissue either by northern analysis or RT-PCR (data not shown). B). rDNA as the loading control. C). Total RNA from the adult brain, dissected into three distinct regions, cerebellum, cerebral cortex and midbrain and from the head of an E17.5 embryo. Expression of p140mRhoGAP was highest in the cerebellum. D). Ribosomal DNA as the loading control.

Figure 11.

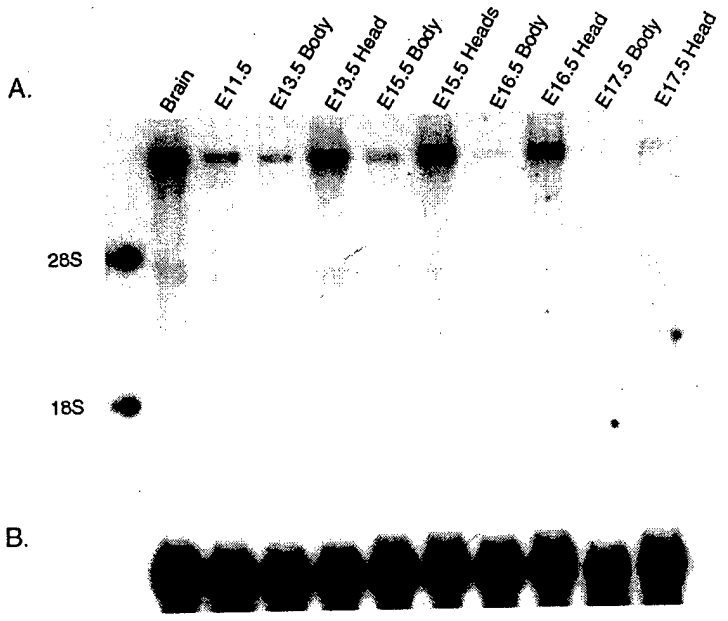

Figure 11. Developmental profile of p140mRhoGAP RNA expression. Embryos from staged pregnancies (appearence of a vaginal plug was considered embryonic day 0.5) were removed and either processed whole for RNA (E11.5) or divided into head and body then processed for RNA (E13.5-E17.5). Expression of p140mRhoGAP is very high in the heads of E13.5 through E15.5 and decreases from E16.5 through E17.5. RNA expression in the bodies of the developing embryos decreases throughout development to birth. The RNA in th eE17.5 body lane is slightly degraded and may contribute to the apparent loss of signal from that sample. B). Ribosomal DNA serves as the loading control.

Figure 12.

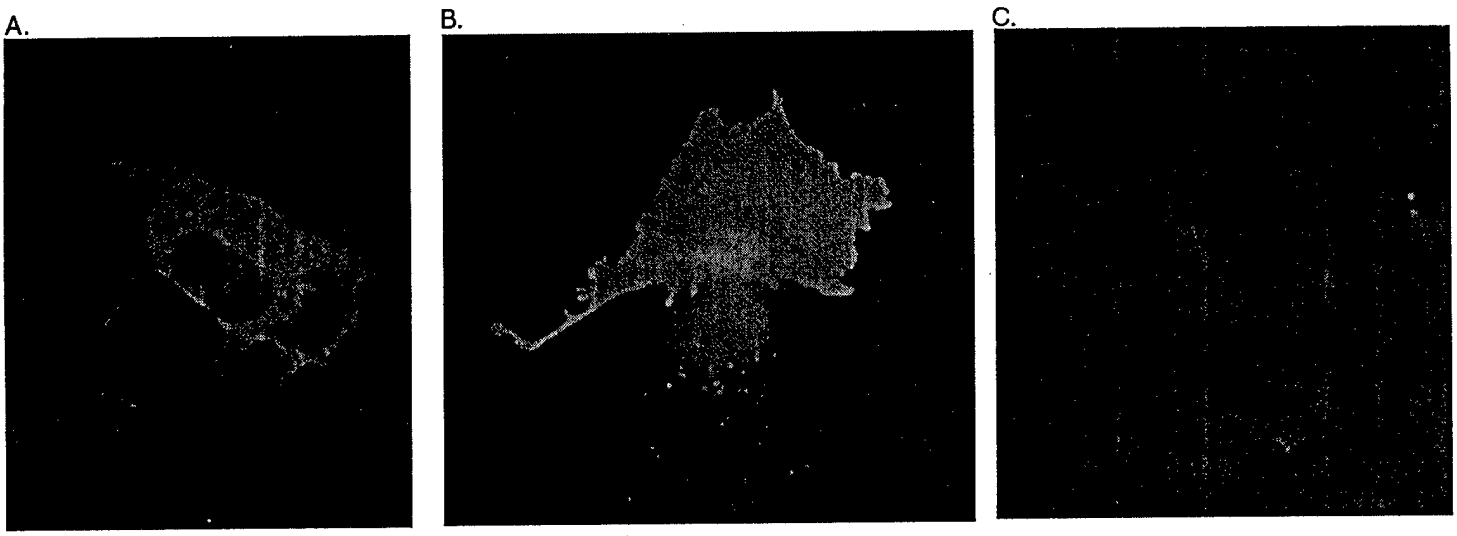

Figure 12. Intracellular localization of p140mRhoGAP. A). The coding region of p140mRhoGAP was fused to the green fluorescent protein (GFP), transfected into COS-7 cells and visualized using optics for fluorescein immunofluorescence. B). The empty GFP containg vector was transfected into COS-7 cells to assess the localization of the GFP moiety. Note that the GFP protein is localized throughout the cell with the heaviest concentration within the nucleus. The p140mRhoGAP fusion protein is definitely not nuclear (compare A and B). C). Untransfected COS-7 cells.

Figure 13.

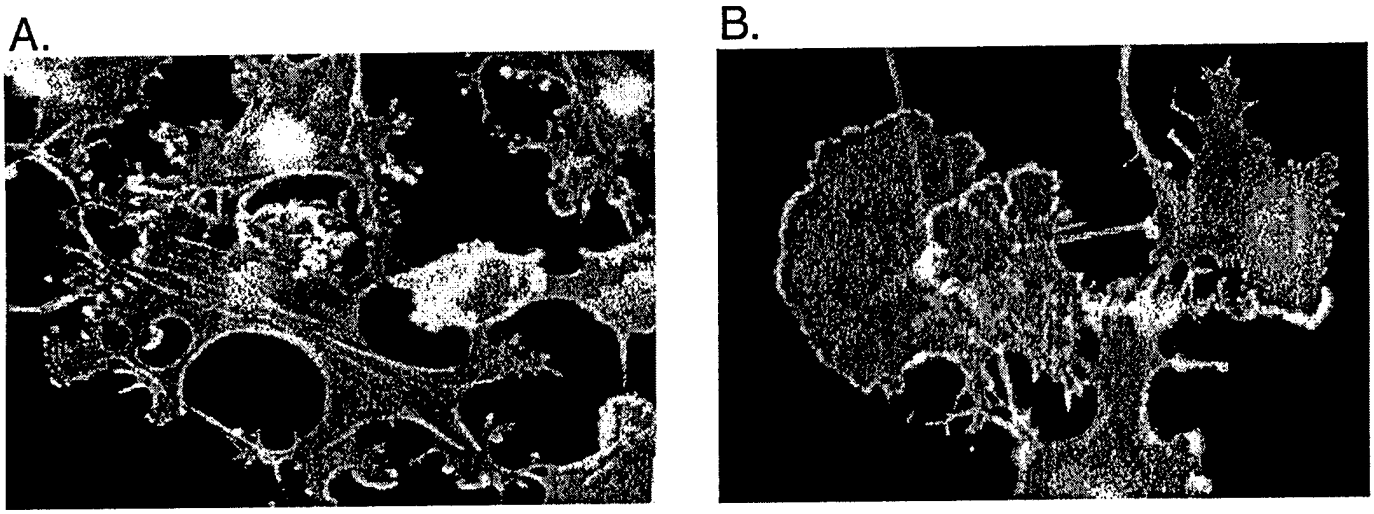

Figure 13. The effects of p140mRhoGAP overexpression on stress fiber formation in COS-7 cells. COS-7 cells were stably transfected with the full length p140mRhoGAP cDNA under the control of the CMV promoter. A). Stress fiber formation in the empty vector control transfectants. Cells were grown on coverslips in the presence of serum for 24 hours, serum was removed and the cells continued to grow for and additional 56 hours after which serum was replaced in the media for 4 hours. Stress fiber formation has been shown to be dependent on Rho activation (Ridley, 1996). B). COS-7 cells expressing p140mRhoGAP. The cells were treated exactly as described for the vector only transfectants. Stress fiber formation in the p140mRhoGAP expressing cells is inhibited or severely reduced possibly as a result of Rho downregulation by RhoGAP. All cells were stained with Rhodamine-Phalloidin for actin filaments as described in (Tribioli, et al. 1996).